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L-Carnitine enhances resistance to oxidative stress by reducing DNA damage in *Ataxia telangiectasia* cells

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Abstract

In this study, the modulating effect of L-carnitine on *tert*-butyl-hydroperoxide-induced DNA damage was compared with that of mannitol, a well known scavenger of hydroxyl radicals, both in normal and *Ataxia telangiectasia* mutated (ATM)-deficient lymphoblastoid cell lines established from *A. telangiectasia* (A-T) patients. The alkaline version of the comet assay was employed to measure the frequency of single-strand breaks (SSBs) and alkali-labile sites induced by *t*-butyl-OOH immediately after treatment and at different recovery times in normal and A-T cell lines, with and without pre-treatment with L-carnitine. In addition, both the yield of induced chromosomal damage and the effect on cell proliferation were evaluated. Our results show that pre-treatment of cells with L-carnitine produced an enhancement of the rate and extent of DNA repair in A-T cell lines at early recovery time; furthermore, in samples pre-treated with L-carnitine a reduction of all types of chromosomal aberration was observed, both in A-T and in wild-type cell lines. The reducing effect of L-carnitine pre-treatment on oxidative DNA damage was more prominent than that of pre-treatment with mannitol. In conclusion, we demonstrated a protective effect of L-carnitine on oxidative stress-induced DNA damage in A-T cells, suggesting its possible role in future pharmacological applications in A-T therapy.

Keywords: *Ataxia telangiectasia*; Oxidative stress; L-Carnitine; DNA repair; Chromosomal damage

1. Introduction

DNA damage is one of the most critical threats to cellular homeostasis and life. In order to activate multiple signalling pathways at once, sophisticated sensing and transduction systems evolved to convey the damage signal simultaneously to multiple effectors. The ATM (*Ataxia telangiectasia* mutated) protein plays a central role in such a system. This protein kinase, identified as the product of the gene that is mutated (lost or inactivated) in the human genetic disorder *A. telangiectasia* (A-T) [1], is responsible for sounding the alarm throughout the cell announcing the presence of one of the most cytotoxic DNA lesions, the double-strand break (DSB). After treatment with DNA breaking agents, a fraction of ATM adheres strongly to the sites of the DSBs [2] and tightly controls the initial, rapid phase of the response by phosphorylating important proteins in the G_1/S, S phase and G_2/M checkpoint pathways (reviewed in [3]). Mutations that inactivate wide-ranging regulators such as ATM are assumed to affect many cellular systems and to cause serious disruption of the cellular homeostasis. Indeed, A-T as a disorder is highly pleiotropic and includes the following characteristics: progressive degeneration of the cerebellar cortex, primary immunodeficiency, degeneration of the thymus and gonads, retarded somatic growth, premature aging and acute predisposition to lymphoreticular malignancies. Cells from A-T patients exhibit chromosomal instability and extreme sensitivity to DSB-inducing agents such as ionizing radiation (IR) and radiomimetic chemicals. Importantly, both the repair of DSBs and the signalling of their presence are defective in A-T cells.

Several features of the clinical and cellular phenotypes of A-T, in particular neurodegeneration and premature aging, suggest that defective cells may chronically suffer from increased

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oxidative stress. Support for this hypothesis comes from the observation that ATM-deficient cells are unusually sensitive to the toxic effects of hydrogen peroxide, nitric oxide, and superoxide [4].

Accumulating evidence suggests that increased cellular concentrations of reactive oxygen species (ROS) are important cofactors in tumour promotion [5]. DNA lesions caused by ROS include single- and double-strand breaks, DNA–DNA and DNA–protein cross-links, and base modifications. The main lesions induced by oxidative agents to DNA, for example *tert*-butyl-hydroperoxide (*t*-butyl-OOH) or hydrogen peroxide (H_2O_2) are single-strand breaks (SSBs) [6]. Much less abundant are DSBs [7].

Mammalian cells are endowed with several levels of defence mechanisms against oxidative damage (reviewed in [8,9]), the first of which is based on the direct interaction with ROS and their detoxification by low molecular-weight antioxidants or protein scavengers such as ferritin or ceruloplasmin. An alternative way to decrease the effects of oxidative damage is to administer antioxidants or to enhance the capacity of the cells to repair the damage.

L-Carnitine (β-hydroxy-γ-trimethyl-ammoniumbutyric acid) is a small water-soluble molecule with a key role in mammalian lipid metabolism. It is mainly required in the transport of activated fatty acids from the cytosol into the mitochondrial matrix for subsequent β-oxidation [10]. Mammals acquire their carnitine needs primarily via the diet, even though they are capable to synthesize it endogenously [11]. It has been reported that carnitine protects the myocardium against ischemia [12], myocardial infarction [13], and skeletal muscle myopathy in heart failure [14]. Recent reports have also suggested that carnitine has additional pleiotropic functions in tissues that are not considered lipogenic, such as brain where it has a neuroprotective role [15]. Many studies suggest that L-carnitine could help the cells to repair SSBs induced in DNA [16] and to protect it from oxygen free radicals [17]. Other studies indicate an L-carnitine-dependent reduction of DNA single-strand breaks after *in vitro* treatment with an oxygen radical-generating system, in isolated human lymphocytes [18].

We analysed the modulating effect of pre-treatment with L-carnitine on *t*-butyl-hydroperoxide (*t*-butyl-OOH)-induced DNA damage both in normal and ATM-deficient lymphoblastoid cell lines (LCLs) established from A-T patients with different *ATM* mutations. To understand the mechanism of action of L-carnitine, its effect was compared with that of mannitol, a well-known scavenger of the free hydroxyl radical [19]. As catalase activity has been reported to be low in *ATM*-deficient cells [20], we decided to employ *t*-butyl-hydroperoxide as an inducer of oxidative stress as it is poorly hydrolysed by catalase [21]. The alkaline version of the comet assay, a rapid and sensitive procedure to quantify DNA lesions in mammalian cells [22], was employed to measure the frequency of SSBs and alkali-labile sites induced by *t*-butyl-OOH immediately after treatment and at different recovery times in normal and A-T cell lines, with and without pre-treatment with L-carnitine. In addition, both the yields of induced chromosomal damage and cell proliferation were evaluated.

2. Materials and methods

2.1. Cell lines and cultures

Lymphoblastoid cell lines were established from *A. telangiectasia* patients AT21RM, AT28RM and AT97RM after immortalization with Epstein-Barr virus (EBV). At the molecular level, AT97RM is homozygote for the deletion CCTC at codon 717; AT21RM is homozygote for a deletion of 90 nucleotides at codon 2377 and AT28RM is compound heterozygote for a deletion of a CT at codon 8283 and a truncating substitution C > T at codon 7792 of the *ATM* gene [23]. The cell line FLEBV, presenting a wild-type *ATM* genotype, was used as control. Immortalized LCLs were maintained in RPMI 1640 medium (Gibco) supplemented with 20% inactivated foetal bovine serum (FBS) (Gibco), 2% Hepes, 1% glutamine (invitrogen) and 1% streptomycin and penicillin (invitrogen) at approximately 5×10^5 cells/ml at 37 °C in a humified atmosphere of 95% air and 5% CO_2. Moreover, to eliminate variations due to the culture conditions, control and treated cultures were maintained concurrently and the same batches of culture medium and preparation of chemicals were used for each experiment.

2.2. Chemicals

tert-Butyl-hydroperoxide (*t*-butyl-OOH) [75-91-2], mannitol (D-form) [69-65-8], colcemid [477-30-5] and 5-bromo-2′-deoxyuridine (BrdUrd) [59-14-3], were purchased from Sigma–Aldrich. *t*-Butyl-OOH was diluted in distilled water at the appropriate treatment concentration and used freshly made. In particular, doses of 200 and 500 μM for the analysis of chromosomal damage in the G_2 and G_1 phase of the cell cycle were used, respectively. Mannitol (D-form) was dissolved in distilled water at 1 M, kept frozen at −20 °C until use at a final concentration of 1 mM. L-Carnitine [541-15-1], kindly provided by Sigma–Tau, Italy, was dissolved in Ham's F10 and kept frozen at −20 °C until use at a final concentration of 5 mM.

2.3. Cell treatments with t-butyl-OOH, L-carnitine and mannitol

Briefly, the cell lines were seeded at 5×10^5 cells/ml in RPMI 1640 complete medium and pre-treated with L-carnitine (5 mM) for 48 h or with mannitol (1 mM) for 1 h at 37 °C. Before treatment with *t*-butyl-OOH, the cells were washed twice with phosphate-buffered saline (PBS) and suspended in Ham's F10 complete medium without serum. Cells were exposed to *t*-butyl-OOH for 1 h, washed in PBS to remove the oxidative agent and suspended again in RPMI 1640 complete medium. Treated cells were either used for the comet assay ($t = 0$, 1, 4 h) or incubated at 37 °C for 3 or 24 h to study the chromosomal damage in the G_1 and G_2 phases of the cell cycle, respectively. To the cell cultures harvested at 24 h after *t*-butyl-OOH treatment, 5-bromo-2′-deoxyuridine (BrdUrd) was added (final concentration 6 μg/ml).

The experimental flow chart concerning treatments with L-carnitine plus *t*-butyl-OOH or mannitol plus *t*-butyl-OOH is shown in Fig. 1. All the doses used here were chosen based on pilot experiments.

2.4. Cell-cycle analysis by flow cytometry

To ensure that pre-treatment with L-carnitine or mannitol did not interfere with normal cell-cycle progression, a flow-cytometric analysis was performed both in treated and in untreated samples, immediately after the pre-exposure period. Cells were washed twice with PBS, fixed in cold PBS–methanol 1:1, permeabilized with 1 ml of 0.1% Triton X-100 [9002-93-1] and kept overnight at +4 °C. Cells were then stained with 50 μl of propidium iodide [25535-16-4] solution (1 mg/ml in PBS) containing 0.5 mg/ml DNase-free RNase. The DNA content was analysed by use of a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA) by recording red fluorescence. Analysis of cell-cycle data was performed on 10,000 events, with Cell Quest software.

2.5. SCGE analysis (comet assay)

The standard alkaline (pH > 13) single-cell gel electrophoresis (SCGE), or comet assay, was performed as described earlier under visible fluorescent light